

## An improved immunohistochemical diagnostic technique for canine leptospirosis using antileptospiral antibodies on renal tissue

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**Abstract.** The purpose of this study was to compare the immunoreactivity in canine renal tissues stained with antisera specific for 3 leptospiral antigens and those processed with traditional staining methods. In addition, immunoglobulin staining was done on tissues with immunoreactivity to leptospiral antigens. Formalin-fixed renal sections from 12 dogs with chronic interstitial nephritis suspected or proven to have leptospirosis (6 dogs with silver-stained leptospires and 6 dogs in which silver-stained leptospires were not detected) were used. Antibodies consisted of a monoclonal antibody to *Leptospira kirschneri* serovar grippotyphosa lipopolysaccharide (LPS) and 2 polyclonal antibodies to outer membrane proteins, including OmpL1, a leptospiral porin, and LipL41, an outer membrane lipoprotein. The murine monoclonal antisera against LPS (F71C2-1) had the most abundant and consistent immunoreactivity. Immunoreactive areas were present in 6 of 6 sections positive by silver staining and included extracellular granular debris in intertubular areas, debris in macrophages, organisms in tubular lumina, and cytoplasmic granules in tubular epithelia. Antisera with specificity for the outer membrane proteins OmpL1 and LipL41 detected only intact organisms in tubular lumina. Immunoreactivity to OmpL1 (polyclonal 338) occurred in 4 of 5 sections positive by silver staining, but immunoreactivity to LipL41 (polyclonal 813) occurred in only 1 of 6 silver-positive sections. Each of the kidney sections in which leptospiral antigens were detected by immunohistochemistry also was positive by silver staining. Sections negative by silver staining were also negative by immunostaining. Although immunohistochemistry did not enhance sensitivity, amplification of signal by secondary antibody and hematoxylin counterstaining improved the ease of diagnosis and allowed better evaluation of tissue morphology than did silver staining methods. IgG was the most abundant immunoglobulin. IgG immunoreactivity occurred predominantly in plasma cells within interstitial infiltrates. Interstitial infiltrates contained abundant immunoreactivity to LPS, but immunoreactivity to OmpL1 and LipL41 was not noted.

Leptospirosis, one of the most widespread zoonoses, is a reemerging disease of dogs.<sup>5</sup> Since the first description in 1899, canine leptospirosis in the United States has traditionally been associated with *Leptospira interrogans* serovars canicola and icterohaemorrhagiae. The use of vaccines containing these serovars has markedly reduced the incidence of leptospirosis in dogs.<sup>1,16</sup> In the last decade, leptospirosis caused by *Leptospira kirschneri* serovar grippotyphosa and *L. interrogans* serovars pomona and bratislava has re-

emerged as an important renal and hepatic disease of dogs in North America.<sup>1,4,5,22</sup>

Clinical diagnosis of leptospirosis can be challenging, and multiple concurrent diagnostic methods are often used. Clinical signs are often nonspecific and may include fever, myalgia, anorexia, vomiting, and diarrhea.<sup>17,28</sup> Hematuria and jaundice also may be noted. Serologic tests include the microscopic agglutination test<sup>10</sup> and the enzyme-linked immunosorbent assay.<sup>23</sup> Fluorescent antibody tests,<sup>9</sup> dark field microscopy, culture, polymerase chain reaction (PCR) assays, and histopathology with special stains (e.g., silver) can be used to identify leptospires in the tissues or body fluid of dogs. However, there are drawbacks to each of these diagnostic tests. Serology is complicated by antibody cross-reactivity between different serovars and by the presence of low titers during acute disease.<sup>1,6,22</sup> Previously vaccinated dogs may have elevated titers that further complicate diagnosis, and some dogs may become infected and actively shed organisms without ever having a titer greater than 1:100<sup>29</sup> or may seroconvert to negative after appropriate treatment.<sup>15</sup> Silver stains are often used to identify lepto-

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Received for publication March 5, 2001.

spires in fixed tissues. However, difficulties with this method arise because of extensive reticulin staining and inability to recognize leptospiral fragments, especially if few organisms are present. Darkfield microscopy is an excellent screening tool for urine but is of low sensitivity, and organism shedding can be intermittent.<sup>1,21</sup> PCR assays can be specific and sensitive but are not widely available.<sup>1,15</sup> Culture is the gold standard of diagnosis, but leptospires are difficult to culture and may take many months to grow, and cultures are susceptible to contamination.

Immunofluorescent methods for detection of renal leptospires were developed many years ago<sup>9,19,28</sup> but were performed using whole leptospires or crude preparations.<sup>2,24,27</sup> The 3 antisera used for the present studies were purified murine monoclonal antibody F71C2-1 with specific immunoblot reactivity to the serovar grippityphosa lipopolysaccharide (LPS) antigen,<sup>3,13</sup> polyclonal rabbit antibody with immunoblot reactivity to a leptospiral outer membrane protein OmpL1,<sup>25</sup> and polyclonal rabbit antibody with immunoblot reactivity to a surface-exposed lipoprotein LipL41.<sup>3,26</sup> In recent immunohistochemical and immunoblotting studies, the expression and distribution of specific leptospiral antigens have been characterized during infection in hamsters.<sup>3,20</sup> Although previous studies have addressed the role of immunoglobulin and leptospiral antigen in interstitial inflammatory infiltrates,<sup>3,19</sup> a relationship between immunoglobulin and specific leptospiral antigens has not been described. The purpose of this study was to evaluate the immunoreactivity of these antisera as a diagnostic tool in tissues obtained from dogs with interstitial nephritis and to relate immunoreactivity to specific leptospiral antigens with the presence of immunoglobulins within inflammatory foci.

### Materials and methods

**Dogs.** Cases of suspected leptospirosis ( $n = 12$ ) were retrieved from the archives of the Department of Veterinary Pathology at Iowa State University. Selection was based on a morphologic diagnosis of chronic interstitial nephritis, clinical evidence of acute febrile disease, and documented or suspected leptospirosis. These cases were divided into 2 groups: group 1 included 6 dogs in which leptospires were detected by silver staining of tissue, and group 2 included 6 dogs in which leptospires or leptospiral fragments were not observed in tissue sections. Lesions of interstitial nephritis were subjectively graded according to degree of inflammation that included foci of plasma cells, lymphocytes, and lesser numbers of macrophages and neutrophils:  $-$  = negative,  $+$  = mild,  $++$  = moderate, and  $+++$  = severe and extensive.

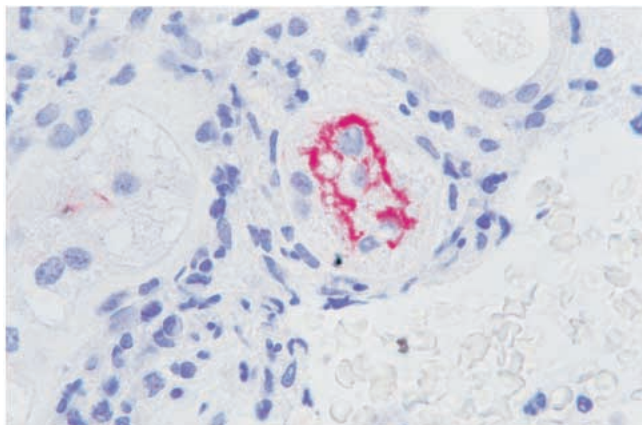
**Immunohistochemistry.** Modifications of previously described immunohistochemical techniques<sup>3</sup> were used. Serial 5- $\mu$ m sections of kidney were placed on positively charged slides<sup>a</sup> and processed using hematoxylin and eosin (HE), sil-

ver stains for leptospires,<sup>8</sup> and 3 antisera for immunohistochemical detection of leptospiral antigens. For immunohistochemical staining, tissues were deparaffinized with xylene, rehydrated through graded alcohols, and incubated in working Tris (pH 7.6) for 15 min at 37 C followed by pretreatment with 0.1% trypsin in 0.1 M Tris HCl (pH 7.6) with 0.1%  $\text{CaCl}_2$  for 5 min at 37 C. Nonspecific staining of tissue sections was blocked using 10% normal goat serum with incubation at room temperature for 20 min prior to incubation overnight at 4 C with primary antibody. Three primary antisera were used at the stated dilutions: monoclonal antibody F71C2-1<sup>b</sup> (1:12,000) to serovar grippityphosa LPS, polyclonal rabbit antibody 813 (1:5,000) specific to the outer membrane protein LipL41, and polyclonal rabbit antibody 338 (1:6,000) specific to the outer membrane protein OmpL1. Description and preparation of rabbit polyclonal antisera to LipL41<sup>26</sup> and OmpL1<sup>11</sup> have been previously published. Controls included normal rabbit serum without primary antibody. Unbound primary antibody was removed by rinsing with Tris, and tissues were incubated at room temperature for 30 min with biotinylated goat anti-mouse immunoglobulin<sup>c</sup> (monoclonal antibody F71C2-1) or biotinylated goat anti-rabbit immunoglobulin<sup>b</sup> (polyclonal antibodies 813 and 338). After washing, sections were incubated for 20 min at room temperature with streptavidin-alkaline phosphatase,<sup>c</sup> and enzyme reactions were developed using a New Fuchsin staining system.<sup>c</sup> Slides were counterstained in hematoxylin for 1 min, dehydrated through graded alcohols and Propar, and coverslipped. Immunoreactivity was graded according to the following criteria: negative ( $-$ ) = no foci,  $+$  = mild,  $<3$  foci,  $++$  = moderate,  $4-9$  foci, and  $+++$  = severe and extensive,  $>10$  foci per histologic renal section.

**Immunoglobulin staining.** Sections were immunohistochemically stained to identify canine IgA, IgG, and IgM in the plasmacytes of chronic inflammatory foci. Tissues were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed on slides preheated to 37 C by microwaving for 1.5 min at 630 W followed by 5 min at 180 W in Tris (pH 10) buffer. Primary antibody was applied at the following dilutions: IgG, 1:30,000; IgA, 1:5,000; and IgM, 1:2,000. Endogenous peroxide activity was blocked by the addition of 3% hydrogen peroxide solution for 2 min. Following 2 rinses in working Tris, secondary antibody<sup>d</sup> (rabbit anti-goat for IgG and IgM and goat anti-rabbit for IgA) was applied for 15 min. An additional 2 rinses were performed then streptavidin<sup>e</sup> was added at a 1:200 dilution and the chromogen 3-amino,9-ethylcarbazole was applied for 20 min. Slides were counterstained with hematoxylin and coverslipped. Immunoreactivity was subjectively assessed as the percentage of reactivity of the total plasma cells for each immunoglobulin.

### Results

Histologic changes in kidneys consisted of interstitial nephritis characterized by multifocal to coalescing infiltrates with plasma cells, lymphocytes, and occasional macrophages and neutrophils located in cortical and medullary areas. In some sections, inflammatory foci also were located in the renal pelvis. Proteina-



**Figure 1.** Immunohistochemical staining of leptospiral antigens with polyclonal 338 (monospecific antisera to OmpL1) is associated with organisms attached to the microvillous surface of a canine proximal convoluted tubule. Immunoreactivity is limited to tubular lumen and is not present in adjacent peritubular tissue, blood vessels, or distal tubule (top right).

ceous precipitates were common in urinary spaces and tubular lumina, and peritubular basement membranes were thickened; some dogs also had evidence of tubular necrosis. In silver-stained sections from dogs 1–6, organisms were present in variable numbers within tubules, and intact and degenerate leptospires were present in proteinaceous tubular casts.

Each case that had silver-stained leptospires also had immunoreactive foci with at least 1 of the antibodies used. Cases that were negative by silver stain also were not immunoreactive. Positive anti-OmpL1 reactivity was restricted in location to the nephron and largely limited to microvillous surfaces of proximal convoluted tubular epithelial cells (Fig. 1). Immunohistochemical staining for LipL41 was observed 1 dog (Table 1) and was associated with intact organisms attached to the microvillous surface of a proximal convoluted tubule. Immunoreactivity was limited to the tubular lumen; it was not present in adjacent peritubular tissue, blood vessels, or distal tubules. In contrast, immunoreactivity to serovar grippityphosa LPS (F71C2-1) was copious and antigens were detected within proximal convoluted tubular epithelium (Fig. 2), in adjacent peritubular connective tissue, and in distal tubular epithelium. In dogs 1, 2, and 5 there were extensive areas within the renal cortex that contained large amounts of leptospiral LPS (Fig. 3). In addition, macrophages in foci of interstitial nephritis contained intense staining for leptospiral LPS antigen (Fig. 4).

The majority (65%) of plasmacytes in areas of inflammation contained IgG. Abundant immunoreactivity to LPS was present in these interstitial infiltrates, but immunoreactivity to antigens associated with intact organisms (OmpL1, LipL41) was not noted. Smaller

**Table 1.** Immunohistochemical reactivity of renal tissue from dogs with chronic interstitial nephritis and acute tubular necrosis. Positive control tissue had positive reactions in all tests; negative control tissues were all negative.

Dog no.	Age (yr)	Sex	Nephritis*	Immunohistochemistry†		
				F71C2	813	338
<i>Silver stain positive for leptospires</i>						
1	5	M	+++	++	—	+
2	3	CM‡	+++	+++	—	NA
3	8	M	+++	+	—	+
4	6	M	+++	+	—	—
5	2	F	+++	+++	—	+
6	5	M	+++	+	+	+
<i>Silver stain negative for leptospires</i>						
7	2	M	+	—	—	—
8	2	M	+	—	—	—
9	NA	CM	+	—	—	—
10	8	F	+	—	—	—
11	7	M	++	—	—	—
12	6	CM	++	—	—	—

\* – = negative; + = mild; ++ = moderate; +++ = severe and extensive.

† Stain amount/intensity; – = negative; + = <3 foci; ++ = 4–9 foci; +++ = >10 foci. F71C2 = monoclonal antibody to *L. grippityphosa* lipopolysaccharide, 1:12,000 dilution; 813 = monospecific rabbit antiserum against outer membrane protein LipL41, 1:5,000 dilution; 338 = monospecific rabbit antiserum to OmpL1, 1:6,000 dilution.

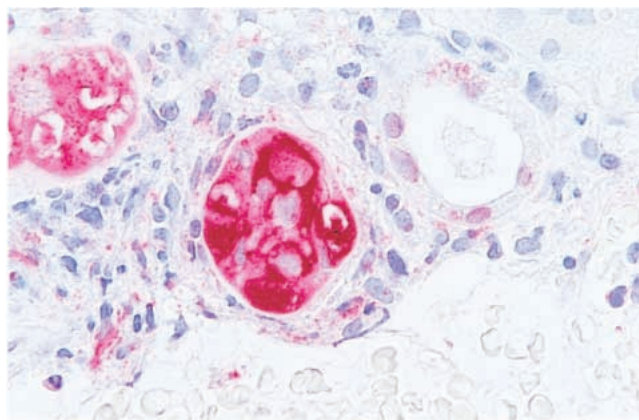
‡ CM = castrated male.

§ NA = information not available.

numbers of plasmacytes stained for IgM. Rare plasmacytes in these foci stained for IgA, although in 4 dogs IgA-positive cells were present.

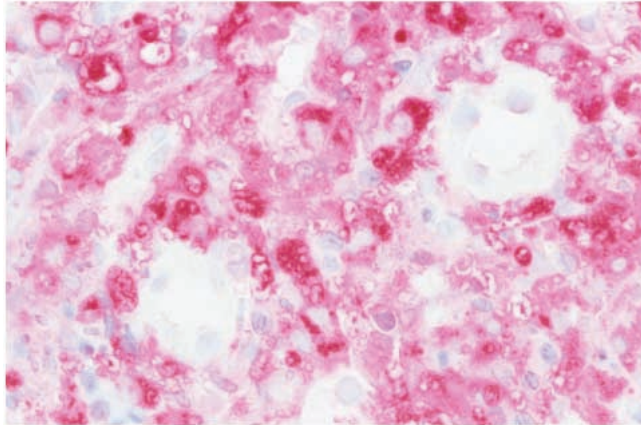
## Discussion

Immunohistochemical reagents prepared against leptospiral antigens are useful aids in diagnosing ca-

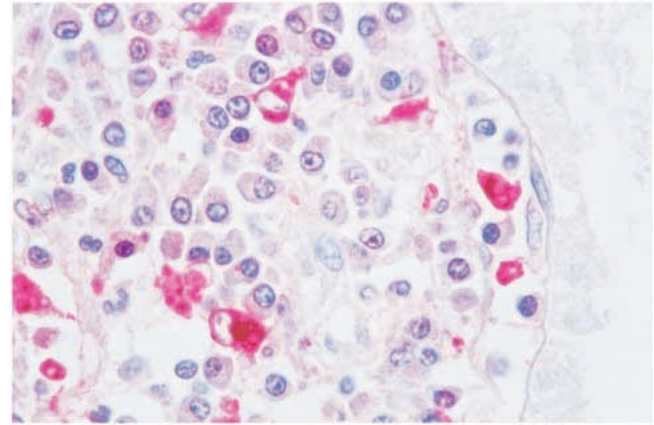


**Figure 2.** Immunohistochemical staining of a section serial to that in Fig. 1 with F71C2-1 (monoclonal antibody to *L. grippityphosa* lipopolysaccharide). Immunoreactivity is abundant within proximal convoluted tubular epithelium, in adjacent peritubular connective tissue, and in distal tubular epithelium.





**Figure 3.** Marked immunoreactivity of antigens in peritubular interstitium of the canine kidney with F71C2-1 (monoclonal antibody to *L. grippityphosa* lipopolysaccharide). Section adjacent to that of Fig. 1. The reactivity is present in macrophages, necrotic cells, and tissue debris.



**Figure 4.** Chronic interstitial inflammatory cell infiltrate with immunoreactivity to F71C2-1 (monoclonal antibody to *L. grippityphosa* lipopolysaccharide). Antigens present in macrophages within a focal area of plasmacytes.

nine leptospirosis. Although the immunohistochemical technique used here did not increase the sensitivity for diagnosis as compared to silver staining methods, tissues stained immunohistochemically were easier to evaluate. The high level of immunoreactivity to the monoclonal antibody for leptospiral LPS in the nephron and surrounding tissue reduces the time required for evaluation, and hematoxylin counterstaining allows for improved tissue evaluation over silver-staining methods. Sites immunoreactive with antisera associated with intact organisms (OmpL1 and LipL41) tended to be few in number, but the red areas of immunoreactivity could be easily distinguished from surrounding tissue. Amount of immunoreactivity to the different antisera may reflect the relative expression of the leptospiral antigens against which the antisera are prepared.<sup>12</sup>

Previous immunoperoxidase staining of renal histologic sections of hamsters with acute tubular necrosis caused by *L. interrogans* serovar canicola had reactivity for intact leptospires and granular deposits in perivascular locations.<sup>20</sup> Only the homologous serovar was agglutinated by monoclonal antibodies, indicating that the recognized epitope is a surface-exposed antigen.<sup>20</sup> Individual serovars may cause different expression of bacterial surface components during replication in vivo. In vivo adaptation by pathogenic leptospires appears to involve a differential expression of outer membrane components, including proteins and LPS.<sup>13</sup> Tissues used in this retrospective study were not cultured for leptospires; therefore, it was not possible to identify which leptospiral serovar was present in immunoreactive cases. However, the murine monoclonal antibody F71C2-1 has specific immunoblot reactivity to serovar grippityphosa. Cross-reactivity to other ser-

ovars is not expected. OmpL1 and LipL41 are antigenically conserved among pathogenic *Leptospira* species,<sup>14</sup> so the presence of intact pathogenic leptospires of any serovar could potentially result in immunoreactivity when using polyclonal antisera 338 or 813.

The presence of leptospiral antigens in intact intratubular locations and within interstitial macrophage cytoplasmic granular debris confirms results of previous studies.<sup>19</sup> Leptospiral antigens have been demonstrated by immunohistochemistry in renal tubules and peritubular macrophages.<sup>2,19,27,28</sup> In most of these studies, the immunofluorescent or immunoperoxidase staining procedures used were associated with loss of tissue structural integrity, resulting in difficulty in assessment of lesion location. In macrophages, intact or discrete leptospires have not been demonstrated immunohistochemically nor have they been seen by ultrastructural examination.

The interstitial inflammatory infiltrate in renal leptospirosis is composed of lymphocytes, monocytes, plasma cells, and occasional neutrophils.<sup>3,7,18,27,28</sup> Lymphoplasmacytic lesions in dogs are strikingly similar to renal lesions of leptospirosis in humans.<sup>27</sup> These infiltrates are thought to function in local production of antileptospiral antibody and phagocytosis.<sup>19</sup> Renal plasma cell populations in canine leptospirosis caused by *L. interrogans* serovar canicola have been previously determined to contain predominantly IgG.<sup>19</sup> In the present study, 65% of the plasma cells contained IgG and 35% of the plasma cells in inflammatory foci were IgM bearing. Immunoreactivity for LPS is abundant in areas of interstitial inflammation where immunoglobulin immunoreactivity occurs. However, it is currently unknown if the immunoglobulin immunore-

activity has a temporal relationship to the progression or resolution of canine leptospiral infections.

Renal immunohistochemistry with formalin-fixed, paraffin-embedded tissues using antisera with immunoblot specificity to leptospiral LPS or outer membrane components is a useful aid in diagnosis of canine leptospirosis from tissues taken by biopsy or necropsy. This technique is an improvement over previous immunohistochemical means of diagnosing leptospirosis in the dog, where antisera were not immunoblot specific and staining procedures resulted in a loss of tissue integrity. Use of this technique in experimental studies could help further define the pathogenesis of canine leptospirosis by pinpointing antigen location at different stages of infection.

### Acknowledgements

We thank Dr. Rudy Hartskeerl for his generous gift of F71C2-1 monoclonal antibody, Ms. Elise Huffman for her excellent technical assistance in immunohistochemical staining, and Dr. Claire Andreasen for her valuable comments and suggestions.

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- b. Dr. Rudy Hartskeerl, Royal Tropical Institute, Amsterdam, The Netherlands.
- c. Kirkegaard and Perry Laboratories, Gaithersburg, MD.
- d. Vector Laboratories, Burlingame, CA.
- e. Zymed, South San Francisco, CA.

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